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Therapeutic Implications of Inhibition versus Killing of *Mycobacterium avium* Complex by Antimicrobial Agents

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Patients with the acquired immune deficiency syndrome (AIDS) with disseminated *Mycobacterium avium* infection have responded poorly to treatment with rifabutine (Ansamycin) and clofazimine, in spite of the good in vitro response of *M. avium* to these antimicrobial agents. We compared the ability of these and other antimicrobial agents to kill versus the ability to inhibit the growth of strains of the *M. avium* complex isolated from patients with AIDS. Killing curve experiments showed that the concentrations of rifabutine and clofazimine needed to kill two log units of *M. avium* are at least 32 times greater than the concentrations needed to inhibit growth. Little or no killing occurred at concentrations of these antimicrobial agents that are achievable in serum. In contrast, five of seven strains tested were killed by ciprofloxacin at concentrations that can be achieved in serum. Ciprofloxacin should be studied further for possible use in the treatment of *M. avium* infections.

It is estimated that about one-half of patients with the acquired immune deficiency syndrome (AIDS) become infected with *Mycobacterium avium* (1). This organism is resistant to most of the antimicrobial agents that are used in the treatment of *Mycobacterium tuberculosis* infections (10, 14). Two antimicrobial agents which show good in vitro activity against most strains of *M. avium* are rifabutine (LM-427; Ansamycin; Farmitalia Carlo Erba Research Laboratories, Milan, Italy) and clofazimine (2, 7, 8, 14). Although clinical trials involving the use of these antimicrobial agents in the treatment of *M. avium* infection in patients with AIDS are not complete, early results of their efficacy have been disappointing (1, 13). The cause of this apparent lack of correlation between in vitro susceptibility results and clinical response is not known. Because of the altered immune status of patients with AIDS, however, effective therapy may require that an antimicrobial agent be bactericidal for *M. avium* and not just inhibitory to its growth. The standard methods that have been used for determining the antimicrobial susceptibility of mycobacteria involve the plating of bacteria on agar media containing the antimicrobial agent (12). These methods can give information about the inhibitory activity of antimicrobial agents but do not give information about their bactericidal action. The recently developed broth tube dilution and radiometric techniques for mycobacteria also yield information about inhibition of growth but not about killing (2, 8). Consequently, most of the information that is available concerning the in vitro susceptibility of *M. avium* complex isolates to rifabutine and clofazimine is based only on the ability of these antimicrobial agents to inhibit the growth of this organism. The purposes of this investigation were to (i) determine whether these or other antimicrobial agents kill *M. avium* complex isolates in an in vitro assay system and (ii) compare the inhibitory and killing levels of antimicrobial agents with achievable serum levels.

Rifabutine was kindly supplied by James Kilburn, Mycobacteriology Branch, Centers for Disease Control, Atlanta, Ga. Clofazimine was a gift from CIBA-GEIGY Corp., Suffern, N.Y. Rifabutine and clofazimine powders were dissolved in a small amount of methanol and then diluted with sterile water. The maximum concentration of methanol in solutions containing antibiotic was 0.64%. This concentration of methanol was not inhibitory to *M. avium*. Ciprofloxacin (Miles Laboratories, West Haven, Conn.), amikacin (Bristol Laboratories, Syracuse, N.Y.), and penicillin (Wyeth Laboratories, Philadelphia, Pa.) powders were dissolved in water. Imipenem (Merck Sharp & Dohme, West Point, Pa.) and gentamicin (Schering Corp., Kenilworth, N.J.) were dissolved in phosphate-buffered saline (pH 7 and 8, respectively). Serial twofold dilutions of all antibiotics were prepared in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) supplemented with ADC enrichment (Difco) and 0.05% Tween 80 (Difco). This broth was used throughout the experiments described here. Tubes containing antibiotic were used immediately, because the activity of antibiotics decreases over time in media used for susceptibility testing of mycobacteria (6).

The strains of *M. avium* complex tested in this study were isolated from blood samples of seven different patients with AIDS. The strains were selected randomly from patients seen at San Francisco General Hospital over a period of several months. One isolate was a serotype 4 strain. Serotypes of the other strains were unknown. Prior to susceptibility testing, bacteria were grown for 7 days in 4 ml of 7H9 broth. The day before the susceptibility test was performed, 0.2 ml of the 1-week-old culture was transferred to 2 ml of fresh 7H9 broth. After overnight incubation at 35°C, bacteria were mixed by inverting the tubes 8 to 10 times. A 0.02-ml fraction of this actively growing culture was transferred to tubes containing 2 ml of 7H9 broth containing antibiotic and to a control tube without antibiotic. This procedure yielded an inoculum of approximately 10^5 CFU/ml in these tubes (mean, 4.1×10^5 CFU/ml; range, 0.8×10^5 to 8.0×10^5 CFU/ml, for seven strains). The actual number of CFU per milliliter in each tube at time zero was determined by plating a 0.1-ml fraction of dilutions prepared in 7H9 broth. Microscopic examination of the inoculum showed little evidence of bacterial clumping.

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TABLE 1. Comparison of MICs and MBC(AFB)s of seven antimicrobial agents against a serotype 4 strain of *M. avium* over time

Antimicrobial agent	MBC(AFB)/MIC (μg/ml) on the following days after drug administration:				
	1	2	3	4	7
Rifabutine	16/ND[a]	8/≤0.03	8/≤0.03	8/≤0.03	ND/≤0.03
Clofazimine	>32/ND	>32/1	>32/1	>32/1	ND/1
Ciprofloxacin	16/ND	1/0.5	1/0.5	1/0.5	ND/0.5
Amikacin	>64/ND	32/4	16/4	16/4	ND/4
Imipenem	>64/ND	>64/0.5	16/0.5	16/1	ND/4
Penicillin	>64/ND	>64/2	>64/2	>64/4	ND/4
Gentamicin	>64/ND	32/8	16/8	16/8	ND/8

[a] ND, Not done.

The inoculated tubes were incubated at 35°C and visually examined daily for turbidity for 4 days and then were examined again after 7 days of incubation. The MIC was defined as the lowest concentration of drug at which the organism did not show visible growth (9). An uninoculated tube of broth was used to make comparisons. Tubes showing no visual turbidity were mixed by inverting them 8 to 10 times. Samples (0.01 ml) were removed from these tubes on each of the first 4 days and spread onto Middlebrook 7H10 agar (Difco) containing OADC enrichment (Difco) and 0.002% WR1339 (Ruger Chemical Co., Irvington, N.J.). Plates were incubated at 35°C for 10 days. Colonies were counted with the aid of a dissecting microscope. The MBC for acid-fast bacilli [MBC(AFB)] was defined as the lowest concentration of antibiotic that killed 99% of the bacterial inoculum. The notation MBC(AFB) is used to distinguish between the definitions used for the MBC for acid-fast versus non-acid-fast bacteria. The predominant colonial morphology on primary plates from patient specimens and in all subcultures used here was the smooth, raised, nearly transparent type. These colonies often had dark spots in the center. This morphology continued to predominate after exposure to antimicrobial agents. Semirough opaque colonies were occasionally seen both before and after exposure to the antimicrobial agents. Thin, smooth, clearly transparent colonies were only rarely seen.

In Table 1 are shown the MICs and MBC(AFB)s of seven antimicrobial agents against a serotype 4 strain of *M. avium*. The low MICs of rifabutine and clofazimine for this strain are consistent with other reports of the inhibitory activity of these antimicrobial agents against *M. avium* (2, 7, 8, 14). In contrast to the MICs, MBC(AFB)s for rifabutine and clofazimine were at least 32 times higher than the MICs. Among the remaining antimicrobial agents tested, after 4 days of incubation ciprofloxacin, gentamicin, and amikacin had MBC(AFB)/MIC ratios of ≤4, while the ratios for imipenem and penicillin were ≥16. Figures 1 to 3 show the kinetics of killing of the serotype 4 strain by rifabutine, ciprofloxacin, and amikacin, respectively. Control tubes containing no antimicrobial agent became turbid after 2 days of incubation and contained colony counts of ca. 10^7 CFU/ml after 2 days and ca. 10^8 CFU/ml after 4 days of incubation. Concentrations of rifabutine of less than 2 μg/ml are not shown in Fig. 1 because the number of colonies on the plates was too large to give an accurate count. The data in Table 1 show that all seven antimicrobial agents included in this study were inhibitory at a concentration that would suggest a therapeutic value. Killing of *M. avium* at an antimicrobial agent concentration that was achievable in serum, however, occurred only with ciprofloxacin and amikacin (and, possibly, with imipenem).

To determine whether the large differences between the MBC(AFB)s and MICs observed with rifabutine and clofazimine were strain dependent, an additional six strains

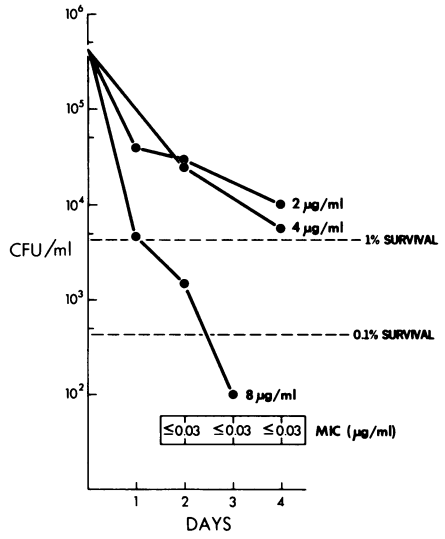

FIG. 1. Killing of an *M. avium* serotype 4 strain with increasing concentrations of rifabutine.

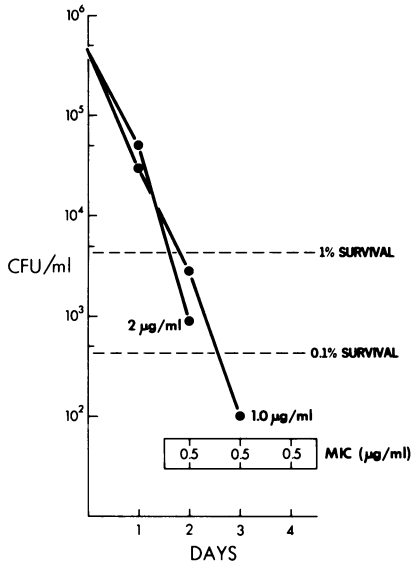

FIG. 2. Killing of an *M. avium* serotype 4 strain with increasing concentrations of ciprofloxacin.

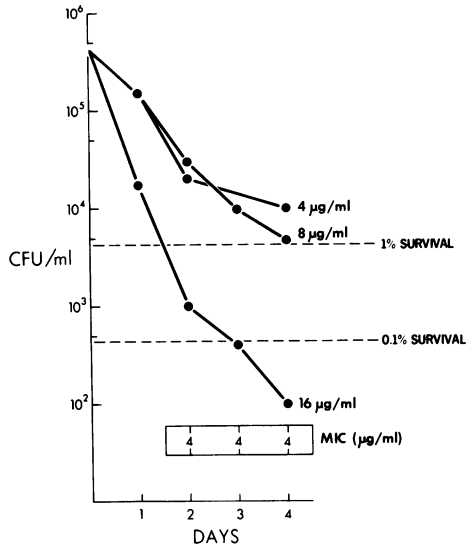

FIG. 3. Killing of an *M. avium* serotype 4 strain with increasing concentrations of amikacin.

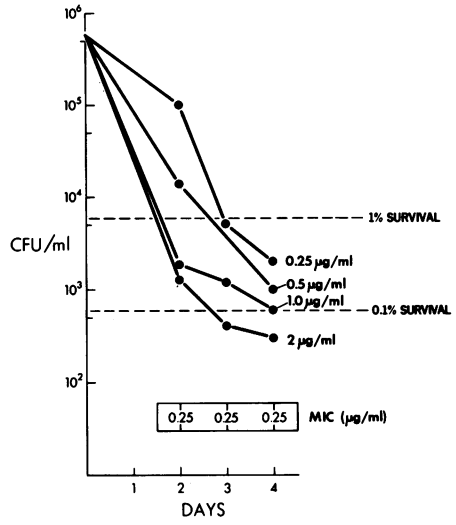

FIG. 4. Killing of a susceptible strain of *M. avium* complex by increasing concentrations of ciprofloxacin.

were tested (Table 2). In each case the killing versus inhibition ratio was ≥32 for rifabutine and clofazimine after 4 days of incubation. These results are in contrast with those obtained with ciprofloxacin in which five of six strains had a killing to inhibition ratio of ≤2. The MICs obtained for ciprofloxacin (Table 2) are similar to the values obtained by Fenlon and Cynamon (4). Killing curves of strains that were susceptible or moderately resistant to ciprofloxacin are shown in Fig. 4 to 5. The inability of clofazimine to cause significant killing of any of the *M. avium* complex strains at concentrations as high as 32 μg/ml is shown in Fig. 6.

The results of these experiments show that while rifabutine and clofazimine are inhibitory to *M. avium* complex isolates in this in vitro assay system, they are not very effective in killing the organism. Levels of rifabutine and clofazimine in serum are approximately 0.5 and 1.0 μg/ml, respectively (8, 15). The experiments described here demonstrate that there is little or no killing of *M. avium* complex strains at these concentrations. Higher concentrations of rifabutine (range, 2 to 32 μg/ml) are required to kill 99% of the organisms in the inoculum. These data are consistent with those of Perumal et al. (11), who reported that a serotype 8 strain could be killed with 5 μg of rifabutine per ml. The inability of rifabutine and clofazimine to kill *M. avium* at achievable levels in serum, combined with the severe immunological impairment seen in patients with AIDS, may help explain why these antimicrobial agents have not been effective in the treatment of *M. avium* infections in this group of patients. The ability of ciprofloxacin and amikacin to kill *M. avium* at achievable levels in serum suggests that these antimicrobial agents should be studied further.

The peak level of ciprofloxacin in serum is approximately 3 μg/ml after a 500-mg oral dose (3). Five of the seven strains of *M. avium* complex tested here had MICs and MBC(AFB)s of ≤2 μg of ciprofloxacin per ml. Effective antimicrobial therapy for disseminated *M. avium* infection in immunocompromised patients probably also requires that the antimicrobial agent is able to penetrate the cells and tissues in which the organism resides. Fong et al. (5) found concentrations of ciprofloxacin in bone and muscle of 1.6 and 2.6 μg/g, respectively, after a dose of 1,000 mg. Experiments are in progress in our laboratory to determine whether ciprofloxacin can kill *M. avium* residing in alveolar macrophages

TABLE 2. MICs and MBC(AFB)s of rifabutine, clofazimine, and ciprofloxacin against six strains of *M. avium* complex[a]

Strain	MBC(AFB)/MIC (μg/ml)		
	Rifabutine	Clofazimine	Ciprofloxacin
3	16/0.12	>32/1	2/2
5	8/≥0.03	>32/1	2/2
8	32/0.06	>32/2	16/4
12	2/0.06	>32/0.5	0.25/0.25
1	2/0.06	>32/1	8/4
11	4/≤0.03	>32/1	0.5/0.25

[a] Values were obtained after 4 days of incubation.

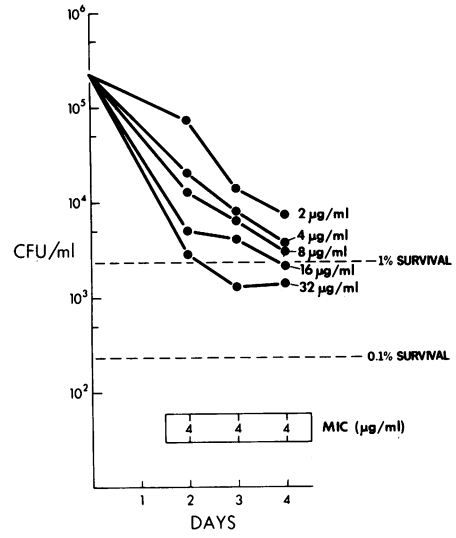

FIG. 5. Killing of a moderately resistant strain of *M. avium* complex by increasing concentrations of ciprofloxacin.

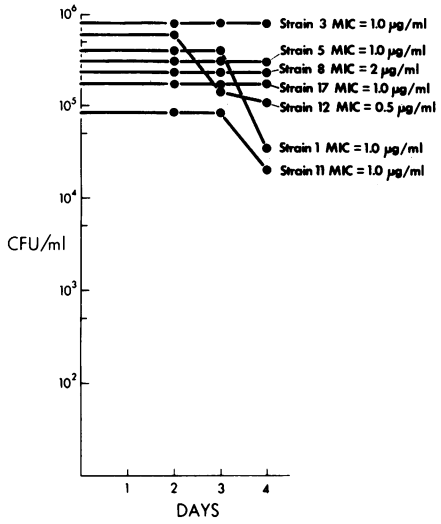

FIG. 6. Survival of seven strains of *M. avium* complex after exposure to 32 μg of clofazimine per ml.

obtained from patients with AIDS. Such experiments may enable us to better predict the efficacy of ciprofloxacin and other antimicrobial agents in the treatment of *M. avium* infections. We are also studying the effect of various components of 7H9 broth on the ability of antimicrobial agents to inhibit or kill *M. avium* in in vitro test systems.

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